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Comparison of Sensitivity Towards Photodynamic Therapy of Cutaneous Resident and Infiltrating Cell Types In Vitro

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Background and Objective: **Photodynamic therapy (PDT) combines photosensitizers absorbing light in the visible spectral region and irradiation with light of corresponding wavelengths. We analysed the sensitivity of cell lines established from resident cutaneous cells and from transformed lymphocytes towards PDT.**

Study Design/Materials and Methods: **PDT was performed employing either 630 or 662 nm light or polychromatic red light (600–700 nm) and photosensitizers Photosan-3, δ-aminolevulinic acid, or methylene blue. Proliferation measured by ^{3}H-TdR uptake was determined in human immortalized keratinocytes (HaCaT) and mouse fibroblasts (NIH/3T3) in comparison to human transformed T-(HuT78) and B-lymphocytes (RA1). Additionally, uptake of the photosensitizers was estimated employing video-intensified fluorescence-microscopy (VIFM).**

Results: **Depending on the photosensitizer tested HaCaT and NIH/3T3 exhibited an ED_{50} up to 10-fold as high as the lymphocytic lines. Polychromatic red light was at least as effective at inducing photodynamic reactions as 630 or 662 nm light. VIFM revealed a positive correlation between sensitivity of a given cell type towards PDT and uptake of the photosensitizers. The differential uptake observed in vitro was confirmed in vivo: A photosensitizer applied topically on a lesion of a patient with mycosis fungoides was found to accumulate preferentially in the lymphocytic infiltrate.**

Conclusion: **Selective topical polychromatic PDT seems to be a feasible goal for the treatment of cutaneous lymphomas.**

Key words: aminolevulinic acid, methylene blue, porphyrins

INTRODUCTION

Photodynamic therapy (PDT) is a promising experimental modality in cancer treatment. It consists of systemic intravenous application of photosensitizers absorbing light of the visible spectral region, e.g. porphyrins, and subsequent irradiation with light of the corresponding wavelength [1]. The photosensitizing porphyrin components accumulate preferentially in tumors rather than in normal tissues [2–4]. Irradiation results in the formation of singlet oxygen which in turn causes tumor regression due to necrosis [5]. This regimen has been successfully applied in the treatment of a variety of solid tumors including skin cancer [6]. Besides, successful application of

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topical PDT has been described in the treatment of cutaneous malignancies [7,8].

Cutaneous T cell lymphomas are lymphoproliferative malignancies mainly of CD4+ T cells [9] for which PUVA is a well established and evaluated photochemotherapeutic approach [10]. This regimen comprises the combination of psoralens as photosensitizers and subsequent irradiation with UV-A light. Besides PUVA, systemic PDT has also been reported to be effective in the treatment of mycosis fungoides [11]. We have recently shown that PDT is capable of inhibiting proliferation of malignant transformed human T cells in vitro and that photodynamic reactions are induced in mycosis fungoides lesions also after topical application of a photosensitizer [12]. In order to further explore the application of topical PDT in this disease we studied the effects on resident cells of the skin and malignant transformed lymphocytes in vitro, testing a variety of different photosensitizers and the effectiveness of 630 nm and 662 nm light as well as polychromatic light (600–700 nm), respectively.

MATERIALS AND METHODS

Cell Lines

T cell line HuT78 [13], derived from the peripheral blood of a patient with adult T cell lymphoma/leukemia, B cell line RA1 [14] from a patient with Burkitt's lymphoma, and the mouse fibroblast cell line NIH/3T3 [15] were purchased from the American Type Culture Collection. Cell line HaCaT derived from spontaneously immortalized human keratinocytes was provided to us by Prof. N.E. Fusenig (DKFZ, Heidelberg, Germany).

Photosensitizers

Hematoporphyrin derivative Photosan-3 (PS3), comparable to Photofrin II, was purchased from Seelab (Deisenhofen, Germany). PS3 is a mixture of different porphyrins including hematoporphyrin IX, protoporphyrin IX, and deuteroporphyrin IX. In aqueous solution, these porphyrins are present as monomers, dimers, or higher aggregates. δ-aminolevulinic acid (ALA) was obtained from Medac, (Hamburg, Germany). This substance, once taken up by cells, is metabolized into protoporphyrin IX which represents the photosensitizing agent when ALA is used. ALA was neutralized to a pH of 7,2 using NaOH immediately before use. Methylene blue (MB) was purchased from Merck (Darmstadt, Germany). The photosensitizers were diluted in medium prior to adding to cell cultures.

Light Sources

630 or 662 nm light was emitted by an Argon-ion laser pumped dye laser (100 mW/cm^2). Light of 630 nm was used in experiments with PS3 and ALA as photosensitizers. In the case of MB the irradiation wavelength was 662 nm. These wavelengths represent absorption maxima of the respective photosensitizers in solution. The 600–700 nm light emitted by a 1200 W bulb (prototype "Waldmann 1200," Waldmann, Villingen-Schwenningen, Germany) was employed for polychromatic irradiation (100 mW/cm^2). The spectral output of this light source in the 600–700 nm range is about constant. The 407 nm line of a Krypton-ion laser served as excitation source for fluorescence measurements.

Cell Proliferation

To determine the effect of PDT on proliferation, 2×10^4 to 2×10^5 cells per well were placed in U-bottom microtiter plates. The photosensitizers were added to final concentrations of 0.1 to 100 μg/ml and the plates were wrapped in aluminum foil and kept in the dark to prevent uncontrolled light exposure. After 2 h of incubation cells were washed three times prior to and after a single exposure to either monochromatic or polychromatic light. Cell cultures not incubated with any photosensitizer were treated similarly. During the final 12 h of a 24 h culture period, ^{3}H-thymidine was added and the uptake was determined. Experiments were done in triplicate. The results indicate the ED_{50}, the dose (J/cm^2) resulting in a 50% proliferation decrease when compared to ^{3}H-thymidine uptake of cells cultured in the presence of the corresponding concentration of photosensitizer without avoidable subsequent exposure to light. Results were readily reproducible at an $ED_{50} > 0.5$ J/cm^2 but not at lower doses due to inevitable exposure to light during handling procedures of the cells already incubated with photosensitizers, e.g. washing.

Fluorescence Intensity, Fluorescence Pattern, Morphological Changes

To visualize the distribution of the photosensitizers within the cells, we used video-intensified fluorescence microscopy with a highly sensitive silicon intensified target camera SIT (Hamamatsu, Japan) as described elsewhere [16]. Fluorescence intensity was measured and classified as negative (−), faint (+), or strong (++). Classifi-

cation was done by two of us (W.H.B. and J.N.) and a third researcher on the basis of coded photographs. In the case of PS3 and ALA, the fluorescence intensity in the spectral range 590–800 nm was detected after excitation with the 405/436 nm bands of a 50 W mercury high pressure lamp, for MB the 530–585 nm band was used. Power densities of 20 mW/cm^2 were applied to limit photochemical modifications during the detection time. The same camera was used to determine morphological changes of the cells during PDT detected by phase contrast microscopy. Measurements were performed after a 2 h incubation time.

In Vivo Fluorescence Pattern

A plaque lesion in a patient with mycosis fungoides was selected for in vivo fluorescence recording. An ointment (Jellin Basis-Salbe, Grünenthal, Stolberg, Germany) containing 10% ALA was applied topically on the selected lesion. The treated area was then covered by a bandage, and after 6 h a biopsy was taken from that lesion. The biopsy was snap-frozen in liquid nitrogen. Fluorescence microscopy was performed on freshly cut and unfixed sections of this specimen.

RESULTS

Proliferation Inhibition by PDT Using 630 or 662 nm Light

The ability of PDT using 630 (with ALA or PS3 as photosensitizer) or 662 nm light (in the case of MB) to inhibit cell proliferation was initially tested on the keratinocyte cell line HaCaT. In the presence of 10 μg/ml ALA a dose of 30J/cm^2 was not sufficient to yield a 50% inhibition of ^{3}H-thymidine uptake (Table 1). The same was true for the fibroblast cell line NIH/3T3, whereas the ED_{50} was found to be profoundly lower for the other cell lines: The respective values were 4.0J/cm^2 in the case of T cell line HuT78 and 10.0J/cm^2 for B cell line RA1.

When PS3 was applied as photosensitizer HaCaT, as the least sensitive cell line again exhibited an ED_{50} of 6.5J/cm^2 in the presence of 10 μg/ml, followed by NIH/3T3 and HuT78 with a comparable ED_{50} (Table 1). In the presence of MB (10 μg/ml) HaCaT remained the least sensitive cell line (Table 1). The relative sensitivities comparing the different cell types were similar in all photosensitizer concentrations tested (data not shown). Irradiation alone or the photosensitizers in the concentrations used caused no or minimal proliferation inhibition (< 5%, data not shown).

TABLE 1. ED_{50} of Cell Lines HACAT, NIH/3T3, HuT78, and RA1 in the Presence of 10 μg/ml Photosensitizer Using Monochromatic or Polychromatic Light*

	ALA	PS3	MB
630/662 nm light:			
HACAT	> 30	6.5	30
NIH/3T3	> 30	0.8	1.3
HuT78	4.0	0.6	2.9
RA1	10	< 0.5	0.8
polychromatic red light:			
HACAT	> 30	2.8	2.8
NIH/3T3	> 30	0.8	0.5
HuT78	0.7	0.8	< 0.5
RA1	4.8	0.9	< 0.5

*Standard deviation was always < 15% at ED_{50} > 0.5 J/cm^2.

Thus, considerable differences were found with regard to the effects of the photosensitizers tested on the different cell types and HaCaT consistently being the least sensitive cell line in the presence of the photosensitizers tested.

Proliferation Inhibition by Polychromatic Red Light

Ultimately, the clinical application of laser light is limited by practical reasons in disseminated dermatoses such as cutaneous T cell lymphomas. Therefore, we studied the effectiveness of 600–700 nm polychromatic light emitted by a 1200 W bulb in inducing proliferation inhibition under similar conditions.

In the case of ALA, the order of sensitivity was similar to that observed with 630 nm light. However, HuT78 and RA1 exhibited a considerably lower ED_{50} (Table 1). In contrast, when PS3 was used, polychromatic red light proved superior only on HaCaT cells. For NIH/3T3 and HuT78, the ED_{50} was comparable to that of 630 nm light (Table 1). In combination with MB, polychromatic red light was more effective when compared with PDT using 662 nm light (Table 1). As 630 or 662 nm light, polychromatic red light alone did not alter ^{3}H-thymidine uptake (data not shown). In conclusion, polychromatic red light seems also to be sufficiently effective in triggering photodynamic reactions in vitro.

Morphological Changes and Fluorescence Pattern

Morphological changes induced by photodynamic reactions were analyzed by phase contrast microscopy: A marked swelling of the cells occurred which rapidly lost their regular shape, and

TABLE 2. Relative Fluorescence Intensity of Cell Lines HACAT, NIH/3T3, HuT78, and RA1 After 2 h Incubation in the Presence of 10 μg/ml Photosensitizer

	ALA	PS3	MB
HACAT	−	+	−
NIH/3T3	+	+	+
HuT78	+ +	+	+
RA1	+ +	+ +	+ +

− = negative
+ = faint
+ + = strong.

TABLE 3. Distribution of the Photosensitizers Tested After 2 h Incubation (10 μg/ml) and Kinetics Under Irradiation

	ALA	PS3	MB
nucleus	−	−	+
cytoplasm	+	+	+
plasma membrane	−	+	+
photobleaching	+	+	−

− = negative (photobleaching: no)
+ = positive (photobleaching: yes).

vacuoles appeared within the cytoplasm (data not shown). This pattern was observed in all but one case: HaCaT cells incubated with MB did not reveal any morphological changes under PDT in the doses used. All cell types cultured in the absence of photosensitizer did not show any morphological changes.

Fluorescence analysis revealed striking differences between the cell types investigated (Tables 2 and 3): ALA induced fluorescence was found within the cytoplasm but neither in the nucleus nor in the membranes. Subsequent irradiation led to rapid and complete decrease of fluorescence, a phenomenon known as "photobleaching." This was true for NIH/3T3, HuT78, and RA1 cells. In contrast, HaCaT showed only minimal fluorescence. PS3 was located within the plasma membrane as well as in the perinuclear cytoplasm and showed photobleaching. All cell types behaved in a similar way. Interestingly, fluorescence in cells incubated with MB was initially very low, and irradiation resulted in an increased intensity throughout the cell. Again, HaCaT was an exception in as much as only very faint fluorescence was detectable which did not increase under light exposure. Cells grown in the absence of photosensitizers did not reveal any detectable fluorescence. With the exception of HuT78 incubated in the presence of PS3 and MB, there was a positive correlation between fluorescence intensity indicating uptake of photosensitizer and sensitivity to PDT. These data indicate that quantitative differences in the uptake of the photosensitizer rather than differences with regard to sensitivity towards photodynamic reactions account for the diverging proliferation inhibition observed in our functional experiments.

Distribution of δ-Aminolevulinic Acid Derived Protoporphyrin IX In Vivo

Having shown considerable differences in vitro with regard to sensitivity and uptake between the cell type tested, we now analyzed the distribution of ALA derived protoporphyrin IX in vivo. ALA was selected because it showed the largest difference in terms of sensitivity between resident and infiltrating cell types in vitro. Six hours after topical application of an ointment containing 10% δ-aminolevulinic acid, fluorescence was found to be most pronounced in skin infiltrating lymphocytes both within dermis and epidermis (Fig. 1). Keratinocytes showed a markedly reduced fluorescence, whereas fibrocytes and endothelial cells exhibited only green autofluorescence (Fig. 1). This finding documents that the differential uptake of this photosensitizer in vitro is reproducable also in vivo. Moreover, the in vitro results on murine NIH/3T3 cells seem to be transferable to human fibroblasts with regard to the low uptake of δ-aminolevulinic acid.

DISCUSSION

To date, the photosensitizers most frequently used for PDT are porphyrins. Their mechanism of action is not fully understood. However, it seems to be clear that the target site for most of these photosensitizers is not the nucleus. Depending on the length of time of exposure damage occurs either at membranes at short exposure or the mitochondria are altered at longer treatments [17]. Recent investigations on mouse lymphoma cells document that PDT utilizing aluminum phthalocyanine as photosensitizer induces rapid apoptosis by phospholipase activation [18,19]. Choosing photosensitizers not targeting the nucleus makes the clinical application of PDT seem favorable over other photochemotherapies such as PUVA, since the risk of inducing malignancies discussed in the context of PUVA [20] could be avoided.

Two other in vivo effects also argue in favor of PDT with porphyrins when compared with PUVA: These photosensitizers show high accumulation in target tissues, where the concentra-

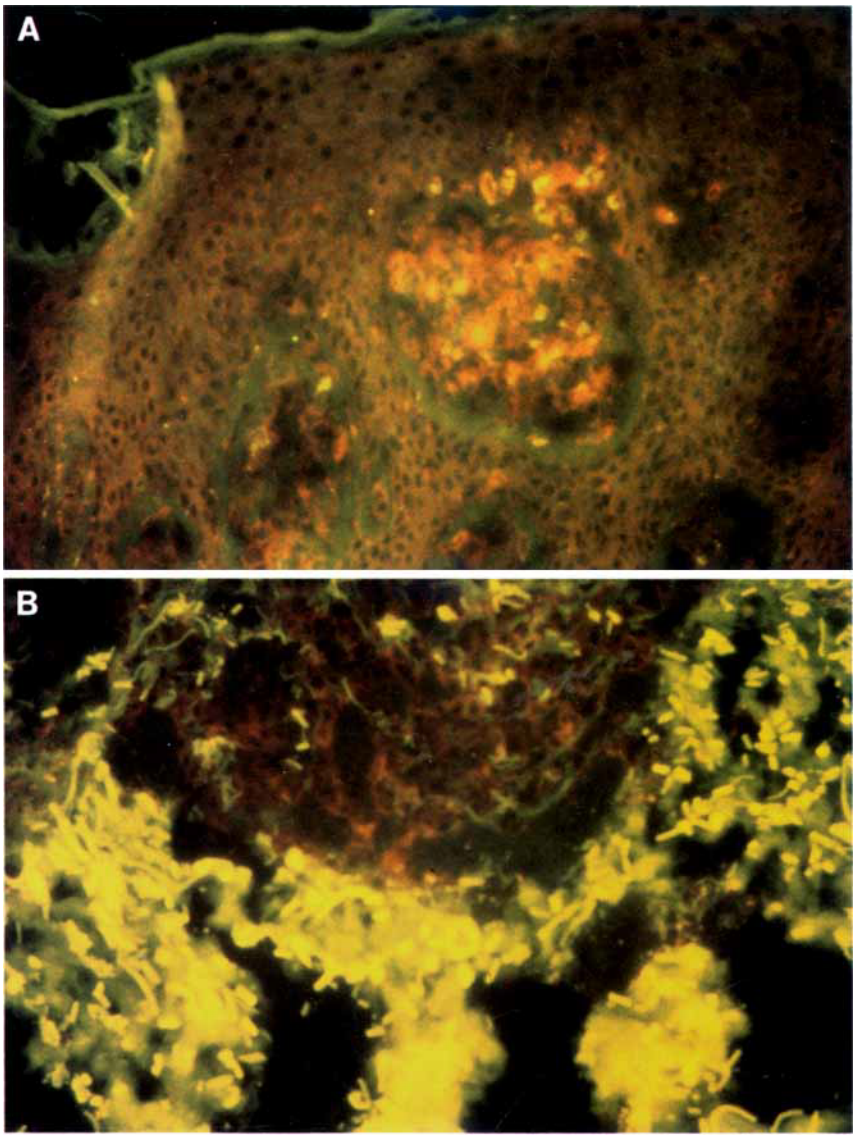

Fig 1. Distribution of δ-aminolevulinic acid derived protoporphyrin IX 6 h after topical application on a plaque lesion of a patient with mycosis fungoides. Note the markedly increased fluorescence of protoporphyrin IX in epidermotropic T cells in comparison to the surrounding keratinocytes (**A**). Within the dermis, formation of protoporphyrin IX is restricted to nests of lymphocytes, whereas the surrounding fibroblasts show green autofluorescence (**B**).

tion of the photosensitizer can reach levels as high as 44 times that of adjacent tissue [2]. Moreover, light of 600–700 nm, which is frequently used for PDT with porphyrins, penetrates tissue much deeper than UV-A light. Thus, higher concentrations of photosensitizers might be reached in the target tissues and photoreactions might occur much deeper within the target lesions.

In mice it has been shown that systemic administration of different photosensitizers and subsequent irradiation of large body areas can cause generalized cutaneous photosensitization and systemic immunosuppression [21]. These severe threats can be avoided if the photosensitizer is applied topically which is possible in cutaneous lesions. We have recently shown that topical application of PS3 results in its uptake into the lesion of cutaneous T cell lymphoma as well as psoriasis [12,22]. This study expands those findings in as much as it demonstrates the selective uptake of the photosensitizer by the lymphocytic infiltrate not only by epidermotropic T cells but also by cells located dermally. The distribution pattern might provide a basis for selective topical

PDT in cutaneous T cell lymphoma and confirms preliminary data of Shanler et al. [23].

There are several pitfalls regarding the utilization of fluorescence intensity as a means to estimate the concentration or absolute amount of a photosensitizer in a cell targeted for PDT. First, there is no simple direct relation between the parameters "fluorescence intensity" and "concentration of photosensitizer." Second, oligomer fractions within photosensitizer mixtures are less fluorescent when compared to monomer fractions but are the components responsible for tumor localization and photosensitization. Moreover, fluorescence recordings via video-intensified fluorescence-microscopy as performed in this study allow semiquantitative interpretations at best. To quantitatively analyze the amount of photosensitizer or its concentration in a target cell other approaches have to be employed. Initial quantitative HPLC analyses in our system have confirmed the qualitative interpretation of our fluorescence data [R. Sailer and W. Straußß, personal communication; A. Rück et al., manuscript in preparation]. These preliminary findings support the concept of cell type dependent differences in uptake of photosensitizers which in turn strongly influence sensitivity towards PDT.

For PDT to be clinically applicable for disseminated dermatoses, suitable light sources are needed. Defocusing the beam of dye lasers frequently used for treating small lesions is very limited. The use of polychromatic light would bypass this limitation. Our data show that polychromatic red light indeed is at least as potent in inducing photodynamic reactions and thus is suitable for PDT. When MB is used as photosensitizer, polychromatic light has a markedly stronger effect. This result is expected since absorption of light differing from the optimal wavelength is still > 0. Additionally, MB is a metachromatic dye showing several absorption maxima in the spectrum of 600–700 nm. Dougherty et al. have demonstrated the effectiveness of polychromatic—however systemic—PDT in a variety of malignant tumors [11]. Our initial observations in psoriasis support the hypothesis that topical polychromatic PDT might also be a safe and effective treatment modality [24].

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